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Recombinant Subunit ORF2.1 Antigen and Induction of Antibody Against Immunodominant Epitopes in the Hepatitis E Virus Capsid Protein

Fan Li,[1,2] Michaela A. Riddell,[1,2] Heng-Fong Seow,[1,3] Naokazu Takeda,[4] Tatsuo Miyamura,[4] and David A. Anderson[1,2]*

[1]*Hepatitis Research Unit, Macfarlane Burnet Centre for Medical Research, Fairfield, Australia*
[2]*Australian Centre for Hepatitis Virology, Melbourne, Australia*
[3]*Department of Medicine, Faculty of Medicine and Health Science, University Putra, Selangor, Malaysia*
[4]*Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan*

A recombinant subunit antigen (ORF2.1), representing the carboxy-terminal 267 amino acids of the 660-amino-acid hepatitis E virus (HEV) capsid protein, was expressed in *Escherichia coli* and used for the immunisation of rats. Purified antigen formulated with either Aluminium Hydroxide Gel Adjuvant (Alum) or Titermax gave high and equivalent levels of antibody after three doses. Responses to two doses of 15, 75, or 150 μg antigen, formulated with Alum and given at 0 and 4 weeks, were also equivalent by 17 weeks after immunisation. Rats initially developed antibody to a wide range of linear epitopes in the ORF2.1 region, but by 27 weeks the predominant response detected by Western immunoblotting was restricted to the conformational epitope unique to ORF2.1 [Li et al. (1997) Journal of Medical Virology 52:289–300], a pattern that was also observed when comparing acute-phase patient serum samples with serum samples from convalescing patients. Antibody from immunised rats blocked the majority of patients' serum reactivity in enzyme-linked immunosorbent assay against both ORF2.1 (57–92% inhibition) and virus-like particles of HEV produced using the baculovirus system (74–97% inhibition). Together, these results suggest that the ORF2.1 subunit vaccine induces an antibody response against immunodominant, conformational epitopes in the viral capsid, which largely mimics that seen in convalescent patients, who are presumed to be immune to HEV infection. ***J. Med. Virol. 60:379–386, 2000.***

KEY WORDS: hepatitis E virus; subunit vaccine; convalescent reactivity; immunodominant epitopes

INTRODUCTION

Hepatitis E virus (HEV) is a single-stranded, positive sense RNA virus responsible for epidemic and sporadic cases of hepatitis E, mainly in developing countries (see Bradley [1995] for review). Sporadic cases of hepatitis E also have been reported in some developed countries [Heath et al., 1995; Kwo et al., 1997], where at least some cases [Schlauder et al., 1998] may represent infection with zoonotic strains of HEV, such as the newly described swine HEV [Meng et al., 1997]. HEV is transmitted by the faecal–oral route, and large epidemics are usually caused by contamination of water. The mortality rate may exceed 21% during pregnancy [Kane et al., 1984], though it is low in the general population.

The HEV genome contains three open reading frames (ORFs), with ORF2 encoding the putative major structural or capsid protein PORF2. The cloning of geographically distinct strains of HEV [Reyes et al., 1990; Huang et al., 1992] and expression of recombinant HEV proteins have led to the development of serologic assays for the detection of HEV infection [Yarbough et al., 1991; Dawson et al., 1992; Goldsmith et al., 1992; Khudyakov et al., 1993; Tsarev et al., 1993; Khudyakov et al., 1994b; He et al., 1995; Li et al., 1997b; Anderson et al., 1999; Li et al., unpublished observations). However, there is currently no licensed hepatitis E vaccine, and although it is clear from passive immunisation that antibody to ORF2 is sufficient to confer immunity [Tsarev et al., 1994], we have a poor understanding of

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*Correspondence to: Dr. David Anderson, Hepatitis Research Unit, Macfarlane Burnet Centre for Medical Research, P.O. Box 254, Fairfield 3078, Melbourne, Victoria, Australia.
E-mail: anderson@burnet.edu.au

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the antigenic structure of the virus and the immune responses important in conferring immunity.

Animal models of hepatitis E infection in nonhuman primate species appear to mirror the pattern of infection, pathologic characteristics, and immune responses seen in human beings [Kane et al., 1984; Bradley et al., 1987]. Swine also have been shown to be susceptible to experimental infection with human HEV strains [Balayan et al., 1990; Meng et al., 1998c], though it appears that some other human HEV strains may be restricted in their host range [Meng et al., 1998b]. Two groups have reported experimental infection of laboratory rats with HEV [Karetnyi et al., 1993; Maneerat et al., 1996], which may provide a more convenient, small-animal model for studies of HEV infection.

Primate models of HEV infection have shown at least partial protective efficacy of experimental vaccines, such as the trpE-C2 fusion protein, representing the carboxy-terminal two-thirds of HEV PORF2 expressed in *Escherichia coli* [Purdy et al., 1993]. Subsequently, immunisation with subviral particles (SVPs) of HEV expressed using the baculovirus system has conferred protection against disease caused by both homologous and heterologous HEV strains in macaques [Tsarev et al., 1994; Fuerst et al., 1996; Tsarev et al., 1997]. Such SVPs, representing amino acids (aa) 112-660 of the capsid protein or with variable C-terminal truncations, are generally thought to mimic the native antigenic structure of the HEV particle [Tsarev et al., 1993; He et al., 1995; McAtee et al., 1996; Li et al., 1997b; Robinson et al., 1998].

Active immunisation against HEV therefore seems feasible, but the relationships between antibody reactivity and immunity to HEV infection are still poorly understood. For example, we do not know whether the antibodies detected by available HEV antibody assays are relevant to the protective immune response. Moreover, there is evidence that at least some antibody specificities may not be cross-protective (in the case of trpE-C2) [Purdy et al., 1993] or may have no neutralising activity at all (in the case of the mosaic HEV protein, consisting of multiple linear epitopes) [Meng et al., 1998a]. The specificity of HEV antibody induced upon immunisation with experimental vaccines is therefore likely to be of greater importance than the total amount of antibody.

In this context, we have sought to identify recombinant antigens that are highly reactive with convalescent-phase sera and conserved between HEV strains, reasoning that such antibodies may be associated with humoral immunity to infection. The results of our previous studies suggest that the carboxy-terminal 267 aa of ORF2 (ORF2.1) contain a conserved, conformational epitope that is strongly reactive with convalescent-phase sera [Li et al., 1994; Li et al., 1997a; Anderson et al., 1999], but it was not clear whether this reactivity represented a major part of the total humoral immune response to the virus. In this study, we examined the antibody specificities induced in rats after immunisation with ORF2.1 antigen produced in *E. coli*, including competitive assays with patients' serum samples. Our results suggest that ORF2.1 can induce a high-titre antibody response to immunodominant epitopes in the HEV capsid (represented by baculovirus-expressed viruslike particles [VLPs]) mimicking that seen in serum samples from convalescent patients. The ORF2.1 antigen therefore represents a promising candidate hepatitis E vaccine.

MATERIALS AND METHODS

Preparation of Recombinant ORF2.1 Antigen

Construction of pQE-AC2.1 Plasmid for Expression. Recombinant ORF2.1 antigen was produced as an insoluble fusion protein with glutathione-*S*-transferase (GST) from the plasmid pGEX-AC2.1 [Li et al., 1994; Li et al., 1997a]. To facilitate the high degree of purification required for a vaccine, the ORF2.1 fragment was subcloned in the vector pQE-30 (Novagen, Madison, WI) to produce a fusion protein with an N-terminal hexahistidine tag. pGEX-AC2.1 was first digested with *Eco*RI, and then the AC2.1 fragment (encoding ORF2.1) was cloned into vector pET-30a(+) (Novagen). The AC2.1 fragment was released from the pET-30a(+) plasmid by digestion with *Bam*HI and *Sal*I and ligated with pQE-30 vector. The resulting pQE-30 plasmid containing AC2.1 was designated pQE-AC2.1. The construction and screening of these clones, as well as examination of fusion protein expression (QE2.1), were performed using methods described previously [Li et al., 1994; Li et al., 1997a].

Production and Purification of QE2.1 Protein. *E. coli* strain JM109, transformed with pQE-AC2.1, was cultured in LB medium containing 100 μg of ampicillin per milliliter for 3–5 hr at 37°C. Isopropylthio-β-D-galactoside was added to a final concentration of 0.5 mmol/L, and the culture was grown for another 5 hr. Cells were pelleted at 3,500 rpm for 15 min in a JA-20 rotor (Beckman Instruments, Fullerton, CA). For cells from each litre of culture, 50 ml of lysis buffer (20 mmol/L Tris-HCl at pH 8.0, 100 mmol/L NaCl) was added, and the pellet was gently resuspended. The suspension was then sonicated in three 10-second bursts and centrifuged at 15,000 rpm for 30 min at 4°C. The pellet was resuspended in 60 ml of lysis buffer containing 8 mol/L urea and incubated at 4°C for 30 min, followed by centrifugation for 30 min as before. The supernatant containing soluble QE2.1 in urea was collected for further purification.

TALON Metal Affinity Resin (Clontech Inc., Palo Alto, CA) was used for purification of QE2.1 in a ratio of 1 ml of resin for 500 ml of culture. The resin was equilibrated with five bed-volumes of lysis buffer containing 8 mol/L urea before the sample was added. The resin/sample suspension was gently agitated at room temperature for 20 min to allow protein to bind to resin and then centrifuged at 500 rpm in a bench-top microcentrifuge (Beckman CS-6) for 5 min. The supernatant was removed, and the resin was washed three times with 10 volumes of lysis buffer containing 8 mol/L urea. The resin was then transferred to a column and

washed twice with 3 volumes of wash buffer (lysis buffer containing 8 mol/L urea and 5 mmol/L imidazole). The purified protein was eluted by adding 4 volumes of elution buffer (lysis buffer containing 8 mol/L urea and 50 mmol/L imidazole). The fractions collected were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The peak fractions were pooled and then refolded by dialysis in 20 mmol/L Tris-HCl, pH 8.0, at 4°C using a dialysis cassette (Pierce, Rockford, IL), with dialysis solution changed every 4 hr. The resulting sample was centrifuged for 15 min at 4°C, and the supernatant was collected. Where indicated, endotoxin was depleted using Detoxi-Gel Endotoxin Removing Gel (Pierce). The protein was quantitated by D_c Protein Assay (Bio-Rad, Richmond, VA) and stored in aliquots at −70°C.

Immunisation of Rats

Wistar rats were 3 months old at the time of immunisation. Purified, refolded QE2.1 was used as a solution in phosphate-buffered saline (PBS) or mixed with Aluminium Hydroxide Gel Adjuvant (Alum; Superfos Biosector, Denmark) in a ratio of 50 μg protein per 100 μl Alum and absorbed at 4°C overnight or mixed in a 1:1 ratio with Titermax (Sigma Chemical Company, St. Louis, MO) immediately before immunisation. Immunisations were administered via intramuscular injection of 0.1 ml at a single site. For comparison of adjuvants, three groups of three rats each were immunised via the intramuscular route at 0, 4, and 13 weeks with 50 μg per injection of purified QE2.1 (prepared without endotoxin depletion) in Alum, Titermax, or PBS. Another group of three rats received PBS alone as a negative control. Serum samples were collected from the tail vein every 2 weeks from 6 to 30 weeks and then at week 40 after primary immunisation. To determine the dose of recombinant antigen required to elicit maximal antibody titres, four groups of three rats each were immunised via the intramuscular route at 0 and 4 weeks with 1.5, 15, 75, or 150 μg of endotoxin-depleted QE2.1 absorbed onto Alum. Serum samples were collected every 2 weeks up to 10 weeks and then at weeks 13, 17, and 27 after primary immunisation.

Detection and Characterisation of IgG Anti–Hepatitis E Virus

Western Immunoblotting. Anti-HEV in rat or patient serum samples was detected by Western immunoblotting using GST-ORF2 fusion proteins representing different fragments of full-length ORF2, as described previously [Li et al., 1997a]. Briefly, equimolar amounts of each fusion protein were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with rat or human sera (1:500 dilution). Immune complexes were detected with horseradish peroxidase (HRPO)–conjugated sheep anti-rat IgG or rabbit anti-human IgG (Dako, Denmark) and enhanced chemiluminescence (ECL, UK).

GST-ORF2.1 Enzyme-linked Immunosorbent Assay. Human and rat serum IgG anti-HEV was determined by enzyme-linked immunosorbent assay (ELISA) using recombinant GST-ORF2.1 antigen, as described for human IgG [Anderson et al., 1999] but with replacement of secondary antibodies to avoid cross-reactivity between rat and human IgG in competitive assays. Briefly, serum dilutions (1:300 unless otherwise indicated) were incubated with GST-ORF2.1-coated microtitre plates for 50 min at room temperature and washed. Bound antibody was detected with a 1:6,000 dilution of HRPO-conjugated sheep anti-rat IgG (Amersham) or a 1:5,000 dilution of HRPO-conjugated sheep anti-human IgG (Amersham) and tetramethyl benzidine (TMB) substrate.

Baculovirus Viruslike Particle Enzyme-linked Immunosorbent Assay. Human serum IgG anti-HEV was also determined by ELISA using purified VLPs of HEV (0.1 μg/well) produced using the baculovirus system, as previously described [Li et al., 1997b] but with substitution of secondary antibody, as mentioned earlier.

Competitive Enzyme-linked Immunosorbent Assay. To determine whether the antibodies induced by QE2.1 in rats were reactive against a range of epitopes similar to those found in HEV patients, competitive ELISAs were performed with both GST-ORF2.1 and VLP-coated plates. Rat sera (1:100 final dilution of pooled or individual pre- or postimmunisation rat sera) and human sera (1:300 final dilution of acute or convalescent patients' sera) were added simultaneously into duplicate ELISA wells and processed as describe earlier for the detection of bound human IgG. The anti-human IgG was shown to have no cross-reactivity against rat IgG (results not shown). Serial twofold dilutions of international reference standard HEV serum 95/584 (100 IU per ml) (a gift from Morag Ferguson, National Institutes for Biological Standards and Control, UK) were assayed in triplicate wells on each plate, to construct a standard curve for the levels of bound human anti-HEV IgG, as described in detail elsewhere [Anderson et al., 1999]. The rate of inhibition was calculated using the quantitative antibody units of patients' samples when assayed in the presence of pre- and postimmunisation rat sera. Except where indicated, samples were assayed on two separate occasions.

RESULTS

Dynamics of Antibody Responses to QE2.1

Comparison of Adjuvants. To evaluate the immunogenicity of QE2.1, groups of three rats each were immunised with either soluble QE2.1 or QE2.1 formulated with either Alum (the only adjuvant licensed for human vaccines) or Titermax (a synthetic adjuvant that has efficacy similar to Freund's complete adjuvant) at 0, 4, and 13 weeks. Figure 1 shows the dynamics of antibody responses to QE2.1 as detected by ELISA with GST-ORF2.1 antigen. High levels of anti-

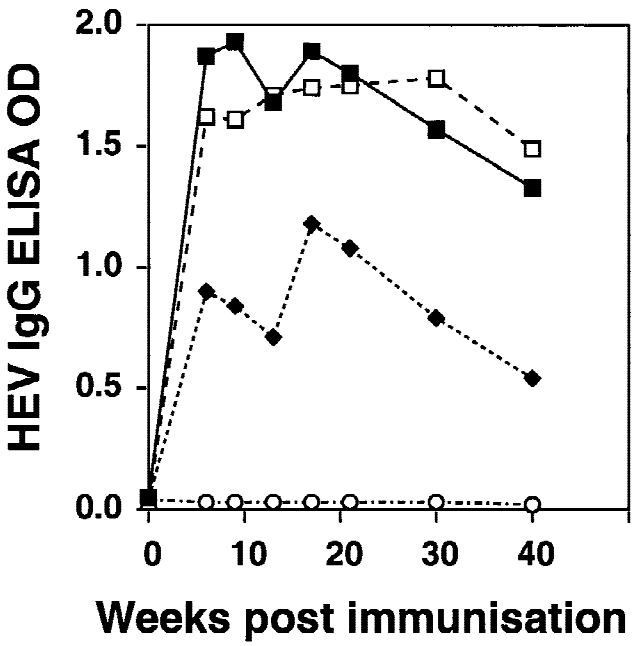

Fig. 1. Kinetics of antibody responses to QE2.1 with different adjuvants. Groups of three rats each were immunised at 0, 4, and 12 weeks with 50 μg of QE2.1 with Alum (■———■), Titremax (❑-----❑), or PBS (♦ · · · ♦) or with phosphate-buffered saline alone (○— · ○). Serum samples were tested for hepatitis B virus–specific IgG by GST-ORF2.1 enzyme-linked immunosorbent assay, and the mean optical density for each group of rats is shown for each sample time.

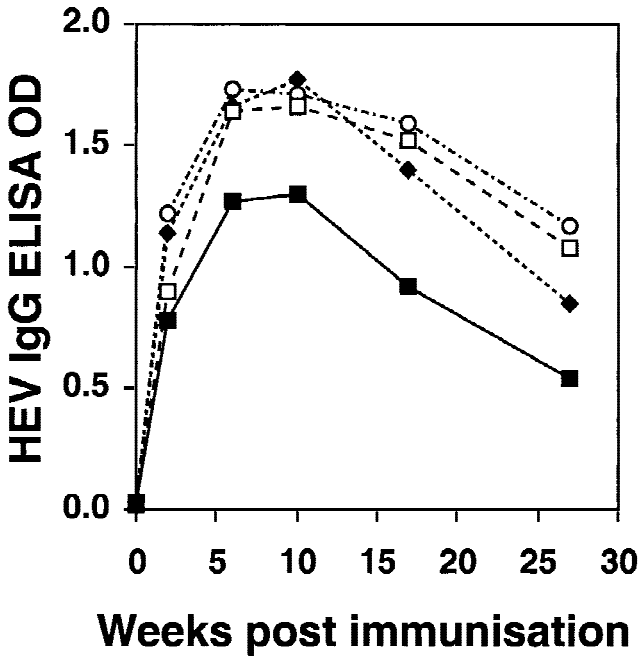

Fig. 2. Kinetics of antibody responses to different doses of QE2.1 with Alum adjuvant. Groups of three rats each were immunised at 0 and 4 weeks with 1.5 (■———■), 15 (❑-----❑), 75 (♦ · · · ♦), or 150 μg (○– · – ·○) QE2.1 with Alum. Serum samples were tested for hepatitis E virus–specific IgG by GST-ORF2.1 enzyme-linked immunosorbent assay, and the mean optical density for each group of rats is shown for each sample time.

HEV were detectable in animals receiving QE2.1 with Alum or Titermax by 6 weeks (2 weeks after the first booster dose), whereas moderate levels of antibody were induced by QE2.1 alone. Levels of antibody were increased after the second booster at 12 weeks but subsequently declined in each group. Nevertheless, it is clear that high levels of anti-HEV were maintained up to 40 weeks after immunisation, which demonstrates that QE2.1 formulated with aluminium hydroxide as the adjuvant is able to induce a strong and lasting immune response.

Titration of Antigen Dose. To determine the optimal dose of QE2.1 for immunisation of rats, groups of three animals each were immunised with 1.5, 15, 75, or 150 μg QE2.1 with Alum at 0 and 4 weeks, and serum samples were tested at intervals for anti-HEV (Fig. 2). Anti-HEV was detectable at 2 weeks after initial vaccination in all animals receiving Alum-conjugated vaccine; the antibody level continued to increase until it peaked at approximately week 10 and declined somewhat thereafter. Table I shows the reciprocal end-point titres of individual rat serum samples in each of the QE2.1/Alum dose groups at weeks 6 and 17. At week 6 (i.e., 2 weeks after boosting), specific IgG titres were higher with increasing dose of antigen. However, at week 17 (13 weeks after boosting), when the specific IgG titres were gradually declining, there was no clear difference between the groups immunized with ≥15 μg of antigen. These results indicate that two doses of 15 μg of QE2.1 formulated with aluminium hydroxide are sufficient to induce high titres of anti-HEV in rats.

Characterisation of Antibody Responses to QE2.1

Western Immunoblotting. We have previously reported that the addition or deletion of as few as 20 HEV-specific aa from the N-terminal part of ORF2.1 ablates its reactivity with convalescent macaque and patient serum samples [Li et al., 1997a]. As such, the reactivity pattern of these truncated or extended recombinant HEV proteins can be used to probe the specificity of antibody responses (Fig. 3). In the present study, 12 fusion proteins from the series were electrophoresed in replicate SDS-PAGE gels and transferred to nitrocellulose, and the membranes were probed with sera from patients in the acute or convalescent phase of illness or with sera collected at 2, 10, or 27 weeks after primary immunisation from rat 7 (immunised with 75 μg of QE2.1 with Alum, as in Fig. 2 and Table I). Human acute-phase serum reacted to an approximately equal extent with all the fusion proteins (Fig. 3A), whereas the convalescent-phase serum was exclusively reactive with GST-ORF2.1 (Fig. 3B), as we have observed previously [Anderson et al., 1999].

Significantly, the serum samples from the immunised rat show a similar dynamic change in the pattern of antibody responses (Fig. 3C–E). At 2 weeks after primary immunisation (Fig. 3C), antibodies were de-

TABLE I. Dose Response of QE2.1/Alum Vaccine in Rats

Antigen dose	Rat no.	Reciprocal end-point titre	
		Week 6	Week 17
2 × 1.5 μg	1	6,400	1,600
	2	6,400	1,600
	3	6,400	1,600
2 × 15 μg	4	25,600	12,800
	5	25,600	6,400
	6	12,800	12,800
2 × 75 μg	7	25,600	12,800
	8	12,800	3,200
	9	51,200	3,200
2 × 150 μg	10	51,200	12,800
	11	51,200	12,800
	12	51,200	12,800

tected against epitopes in the region between 2.1-1 and 2.1. Since they are detected equally well in all larger fusion proteins (containing additional HEV sequences not present in the vaccine), it can be assumed that they are directed against linear epitopes in this region. Similarly, at 10 weeks (Fig. 3D) antibodies against linear epitopes throughout 2.1 were detected, but it is notable that reactivity against GST-ORF2.1 is already predominant. At 27 weeks, reactivity is almost exclusively against ORF2.1, in a similar pattern to that of human convalescent-phase sera, and is thus directed to the conformational 2.1 epitope that is formed after refolding of ORF2.1 in either immunoblot (Li et al., 1997a) or ELISA formats (Anderson et al., 1999). Similar patterns of responses were seen in all rats examined (results not shown).

Competitive Enzyme-Linked Immunosorbent Assay. Although the previous data imply that the humoral immune response generated in rats after immunisation with QE2.1 at least partly mimics that seen in HEV-infected patients, it is not known what proportion of the total humoral immune response actually is detected by Western immunoblotting. Accordingly, competitive ELISAs using GST-ORF2.1 or baculovirus-expressed VLPs were performed to examine the capacity of HEV-specific antibodies induced in rats to compete with HEV-specific antibodies in sera from patients or an experimentally infected chimpanzee. Test serum samples were mixed with a pool of pre-immunisation rat sera or pooled 9- to 11-week sera from rats immunised with 3 × 50 μg QE2.1 with Alum (as in Fig. 1) and were assayed in duplicate on both GST-ORF2.1 and baculovirus VLP ELISAs. The level of inhibition of human IgG binding by immune versus pre-immune rat sera is shown in Table II. Inhibition by immune rat sera of acute- and convalescent-phase human and chimpanzee sera was observed in all samples, in both GST-ORF2.1 and baculovirus VLP ELISAs, with the inhibition rate ranging from 57% to 97%. There appeared to be no relationship between the level of inhibition and the initial level of patient IgG reactivity, the stage of infection, or the strain of HEV. The strongest inhibition observed was with highly reactive, convalescent-phase serum from a patient infected with the Mexico strain of HEV, the most divergent from the Chinese strain.

In addition, individual serum samples from rats immunized with two (n = 9) or three doses (n = 3) of QE2.1 were tested with a single convalescent-phase patient serum sample (GS31) in competitive baculovirus VLP ELISA. Sera collected at 27 weeks from rats immunized with two doses of 15–150 μg QE2.1 inhibited baculovirus VLP ELISA reactivity of GS31 by 34% to 79% (mean, 63%; SD, 14%), whereas week 30 sera from rats immunized with three doses of 50 μg QE2.1 inhibited reactivity by 81–86% (mean, 83%; SD, 2%). These results show that levels of competitive antibodies remain stable between weeks 9 and 11 (Table II, before the second booster dose) and week 30 in rats receiving 3 × 50 μg QE2.1, mirroring the level of reactivity by GST-ORF2.1 ELISA, but they also suggest that three doses of QE2.1 may induce higher levels of competitive antibodies than two doses.

DISCUSSION

The well-controlled passive-immunisation study of Tsarev and colleagues [1994] has clearly shown that antibodies can confer immunity to HEV infection, and a number of studies have shown the efficacy of SVPs in active immunisation [Tsarev et al., 1994; Fuerst et al., 1996; Tsarev et al., 1997]. SVPs and VLPs therefore are considered to be suitable substitutes for "native" HEV antigen. We have now shown that recombinant QE2.1 protein induces an antibody response in rats with specificities similar to those in HEV-infected patients and chimpanzees, as measured by competitive ELISA using baculovirus-expressed VLPs. Furthermore, this antibody response appears to be directed largely against the conformational ORF2.1 epitope and persisted at high levels for at least 9 months. As such, QE2.1 can be considered an alternative antigen for active immunisation against HEV infection, with potential advantages related to the physical characteristics and methods of expression and purification of the protein.

Recent data have suggested that antisera to linear HEV epitopes have no neutralising activity, whereas patient sera are strongly neutralising in an HEV/cell-binding assay [Meng et al., 1998a]. Similarly, baculovirus-expressed SVPs appear to be more effective as candidate vaccines than the trpE-C2 fusion protein, though both induce high levels of total anti-HEV [Purdy et al., 1993; Tsarev et al., 1994; Fuerst et al., 1996; Tsarev et al., 1997]. We therefore think that it is important to consider the specificity of antibodies induced by putative HEV vaccines rather than only the total amount of reactive antibody; available evidence points to the importance of conformational epitopes in the capsid protein. We believe that the current study is the first to examine the specificity of immune responses in animals immunised with an experimental HEV vaccine.

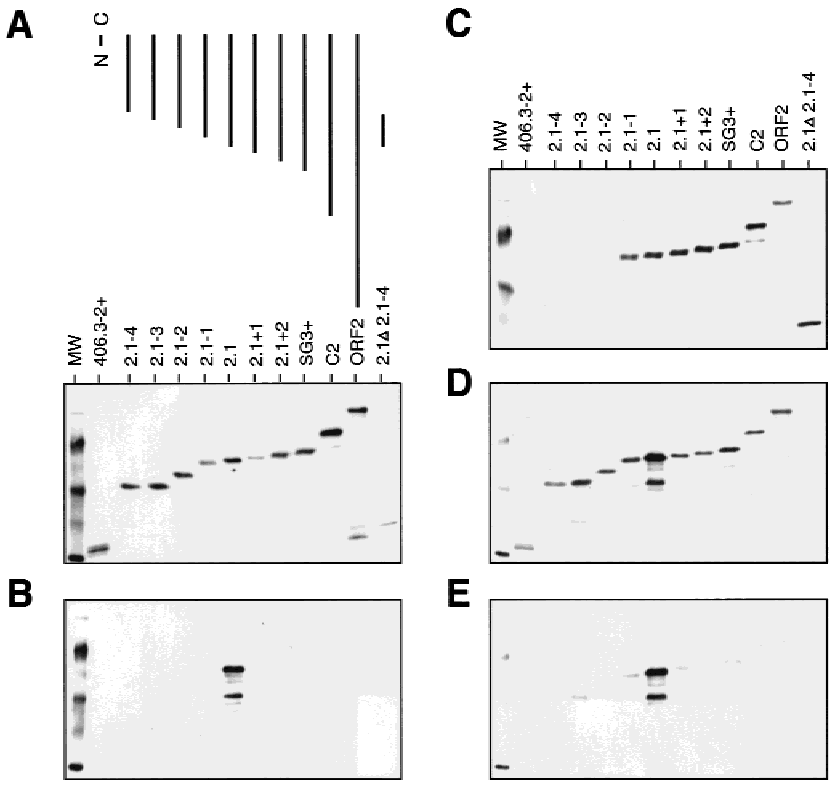

Fig. 3. Specificity of antibody in patients and in an immunised rat against recombinant hepatitis E virus (HEV) antigens by immunoblotting. Serum samples from HEV–infected patients or from a rat immunised with 75 μg QE2.1 at 0 to 4 weeks were used to probe recombinant GST-HEV fusion proteins, representing N-terminal truncations or extensions of HEV sequences relative to ORF2.1 (aa 394-660) as follows: 406.3-2+, 613-660; 2.1-4, 474-660; 2.1-3, 457-660; 2.1-2, 434-660; 2.1-1, 414-660; 2.1+1, 374-660; 2.1+2, 356-660; SG3+, 334-660; C2, 225-660; ORF2, 1-660; 2.1Δ2.1-4, 394-473. **A:** Acute-phase patient and diagrammatic representation of the ORF2 fragments above the corresponding lanes. **B:** Convalescent-phase patient. **C–E:** immunised rat at 2 (C), 10 (D), or 27 weeks (E) after primary immunisation.

TABLE II. Competitive Inhibition of Anti-HEV Reactivity by Antibodies Present in the Sera of Rats Immunised With QE2.1 Vaccine*

Serum	Phase of infection[a]	HEV source	HEV IgG (mIU/ml)[b]	Immune rat serum inhibition (mean % ± SD)[c] GST-ORF2.1 ELISA	Immune rat serum inhibition (mean % ± SD)[c] Baculovirus VLP ELISA
A1	Aucte	India	91	72 ± 3	95[d]
GS7	Acute	Nepal	39	78 ± 0	86 ± 2
GS17	Acute	Nepal	24	64 ± 4	81 ± 4
GS56	Acute	Nepal	111	80 ± 4	89 ± 1
GS16	Convalescent	Nepal	95	69 ± 1	85 ± 3
GS26	Convalescent	Nepal	46	58 ± 0	76 ± 3
GS31	Convalescent	Nepal	49	72 ± 1	87 ± 3
GS37	Convalescent	Nepal	26	57 ± 5	86 ± 1
N158[e]	Acute	Mexico	46	72 ± 3	82[d]
N116	Convalescent	Mexico	173	92 ± 4	97 ± 1
N162[e]	Convalescent	Mexico	51	73 ± 2	74 ± 3

*HEV, hepatitis E virus; ELISA, enzyme-linked immunosorbent assay; VLP, viruslike particles.
[a]Acute <6 months; 1 year ≥ convalescent ≥ 6 months.
[b]Measured in the GST-ORF2.1 ELISA in the presence of pre-immune rat sera.
[c]Inhibition of test serum reactivity in indicated ELISA by serum from immunised rat. Mean of two experiments is shown.
[d]Tested only once.
[e]Experimentally infected chimpanzee.

During the first 2 weeks after primary immunisation with QE2.1, the antibodies produced were reactive with epitopes in GST-ORF2 (ORF2 aa 1-660) and various truncated proteins, including those spanning aa 394-473 (2.1Δ2.1-4) and 414-660 (2.1-1) (Fig. 3C). Weak reactivity to 2.1-2 was also detected in some rats 2 weeks after immunisation (results not shown). These data suggest that linear B-cell epitopes in the region 414-457 are recognised during the early phase of the immune response. At week 10, additional antibodies were detected, with the more C-terminal proteins spanning aa 434-660 (2.1-2), 457-660 (2.1-3), 474-660 (2.1-4), and 613-660 (406.3-2+) (Fig. 3D) in a pattern similar to human acute-phase sera (Fig. 3A). This finding indicates that further linear B-cell stimulatory epitopes lie between 457-660 aa, and most likely beyond 474-660 aa, since the protein 2.1Δ2.1-4 was not strongly immunoreactive with rat sera by week 10. These results are in concordance with those of previous experiments using synthetic peptide reactivity with acute-

phase sera from HEV-infected patients, in which linear epitopes were detected at aa 394-470 and 546-580 [Khudyakov et al., 1994a]. Reactivity to all these linear epitopes wanes dramatically by 27 weeks after immunisation (Fig. 3E), as seen for convalescent human sera (Fig. 3B).

While the levels of total anti-HEV IgG fell between weeks 10 and 27, reactivity with ORF2.1 remained high (Fig. 3E), again in a pattern similar to convalescent-phase patient sera (Fig. 3B) and in agreement with our previous finding that the convalescent-reactive epitopes are conformational [Li et al., 1994; Li et al., 1997a; Anderson et al., 1999]. With respect to Western immunoblot reactivity, the antibody response following QE2.1 immunisation therefore mimics the response in humans and macaques infected with HEV.

These data alone, however, cannot address the question of whether the antibodies induced by QE2.1 represent a significant proportion of the total humoral immune response, since it is possible that immunoblotting may not detect all epitope specificities. This question was addressed directly by measuring the ability of anti-HEV from immunised rats to compete with sera from HEV-infected humans and chimpanzees in both GST-ORF2.1 and baculovirus VLP ELISA formats. Strikingly, immune rat sera strongly inhibited the reactivity of patients' sera and primate sera (Table II). This finding clearly demonstrates that QE2.1 can elicit a humoral response that largely mimics the one obtained during HEV infection and further suggests that residues overlapping ORF2.1 (aa 394-660) contribute almost all of the antibody reactivity in VLPs. Because these VLPs represent a further C-terminal truncation from the 112-660 aa expressed in Tn5 cells [Li et al., 1997b], the reactive epitopes can be narrowed to the region from aa 394 (the amino terminus of ORF2.1) to aa 578 or 607, the C-terminus mapped for similar HEV SVPs [Robinson et al., 1998]. The region between aa 112 and 393 of ORF2 appears to contribute little to VLP antibody reactivity. While it is clear that ORF2.1 conformational epitope specificity is very pronounced in the immunised rat sera that show strong inhibition of patient reactivity, at this time we cannot exclude the presence of other epitopes that are presented by QE2.1 and contribute to blocking but are not detected by immunoblotting.

We believe that the QE2.1 antigen has a number of potential advantages as an HEV vaccine. Recombinant protein expression in *E. coli* is simple and inexpensive, and the use of the hexahistidine tag in QE2.1 allows for rapid and convenient purification. Since the QE2.1 protein undergoes complete denaturation in urea during production, yet retains the ability to induce a comprehensive range of antibody specificities, including the conformational epitope, it is reasonable to speculate that the vaccine would be very stable, which has importance in developing countries. Indeed, we have observed no reduction in antibody responses when the QE2.1/Alum vaccine was stored at room temperature for 7 weeks before use in a two-dose regimen (results not shown). Similarly, the robustness of QE2.1 should facilitate the formulation of combination vaccines where buffer conditions may need to be altered to suit other vaccine components.

A general concern with the use of recombinant antigens expressed in *E. coli* is that their tertiary structure does not necessarily resemble that of the native proteins. This may result in the absence of correct conformational B-cell epitopes that may be crucial for the generation of a protective immune response. The results of this study clearly demonstrate that ORF2.1 is an exception: antibodies to the conformational ORF2.1 epitope are readily induced by QE2.1 with Alum adjuvant, and the antibody specificities induced by QE2.1 largely mimic those found in HEV patients. Studies of the protective efficacy of QE2.1 will further define the importance of these antibodies in immunity, since we cannot yet exclude the formal possibility that immunity depends instead on the small proportion of patients' antibodies that were not blocked by immune rat sera in this study.

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